Molecular epidemiology of tuberculosis in Cambodian children

Schopfer, K ; Rieder, Hans L ; Steinlin-Schopfer, J F ; van Soolingen, D ; Bodmer, T ; Chantana, Y ; Studer, P ; Laurent, D ; Zwahlen, M ; Richner, B

Abstract: SUMMARY We analysed Mycobacterium tuberculosis strains from children, hospitalized from January 2004 to July 2008 in the largest paediatric hospital complex in Cambodia. Specimens were tested for drug susceptibility and genotypes. From the 260 children, 161 strains were available. The East African-Indian genotype family was the most common ($59 \cdot 0\%$), increasing in frequency with distance from the Phnom Penh area, while the frequency of the Beijing genotype family strains decreased. The drug resistance pattern showed a similar geographical gradient: lowest in the northwest ($4 \cdot 6\%$), intermediate in the central ($17 \cdot 1\%$), and highest in the southeastern ($30 \cdot 8\%$) parts of the country. Three children ($1 \cdot 9\%$) had multidrug-resistant tuberculosis. The Beijing genotype and streptomycin resistance were significantly associated ($P < 0 \cdot 001$). As tuberculosis in children reflects recent transmission patterns in the community, multidrug resistance levels inform about the current quality of the tuberculosis programme.

DOI: https://doi.org/10.1017/S0950268814001769

Epidemiol. Infect. (2015), **143**, 910–921. © Cambridge University Press 2014
doi:10.1017/S0950268814001769

Molecular epidemiology of tuberculosis in Cambodian children

K. SCHOPFER[1]*, H. L. RIEDER[2,3], J. F. STEINLIN-SCHOPFER[1], D. VAN SOOLINGEN[4], T. BODMER[1], Y. CHANTANA[5], P. STUDER[5], D. LAURENT[5], M. ZWAHLEN[6] AND B. RICHNER[5]

[1] *Institute of Infectious Diseases, University of Bern, Switzerland*
[2] *International Union Against Tuberculosis and Lung Disease, Paris, France*
[3] *Institute of Social and Preventive Medicine, University of Zurich, Switzerland*
[4] *National Mycobacteria Reference Laboratory, National Institute for Public Health and the Environment, Bilthoven, The Netherlands*
[5] *Kantha Bopha Foundation, Phnom Penh, Cambodia*
[6] *Institute of Social and Preventive Medicine, University of Bern, Switzerland*

Received 21 March 2014; Final revision 21 May 2014; Accepted 18 June 2014; first published online 22 July 2014

SUMMARY

We analysed *Mycobacterium tuberculosis* strains from children, hospitalized from January 2004 to July 2008 in the largest paediatric hospital complex in Cambodia. Specimens were tested for drug susceptibility and genotypes. From the 260 children, 161 strains were available. The East African-Indian genotype family was the most common (59·0%), increasing in frequency with distance from the Phnom Penh area, while the frequency of the Beijing genotype family strains decreased. The drug resistance pattern showed a similar geographical gradient: lowest in the northwest (4·6%), intermediate in the central (17·1%), and highest in the southeastern (30·8%) parts of the country. Three children (1·9%) had multidrug-resistant tuberculosis. The Beijing genotype and streptomycin resistance were significantly associated ($P<0·001$). As tuberculosis in children reflects recent transmission patterns in the community, multidrug resistance levels inform about the current quality of the tuberculosis programme.

Key words: Epidemiology, infectious disease, molecular epidemiology, paediatrics, tuberculosis (TB).

INTRODUCTION

The distribution of genotypes in *Mycobacterium tuberculosis* isolates from children provides valuable information on strains that have been circulating most recently in the community. Similarly, the drug susceptibility pattern of strains from children is a better indicator of recent epidemiological developments than that of strains obtained from adults [1–3]. While obtaining such information has great appeal, it is exceedingly difficult to collect specimens from children that allow isolation of *M. tuberculosis*.

We have reported on the implementation of a diagnostic tuberculosis (TB) laboratory service in a large paediatric referral hospital in Cambodia [4]. Here we describe the spoligotype distribution and drug susceptibility pattern profiles of strains isolated from paediatric TB patients. The current investigation aims to provide insight into the epidemiology of TB supplementary to information from resistance surveys obtained from adult patients during the past decade in Cambodia [5–7].

* Author for correspondence: Professor K. Schopfer, Scheuermattweg 43, 3043 Uettligen, Switzerland.
(Email: kurt.schopfer@bluewin.ch)

MATERIAL AND METHODS

Setting

Details of the setting have been described previously [4]. The Jayavarman VII Hospital in Siem Reap is the largest of five Kantha Bopha paediatric referral hospitals in Cambodia. These hospitals cared for about 120 000 hospitalized and 800 000 ambulatory paediatric patients in 2011 [8]. The mycobacteriology service, established in the Siem Reap hospitals, also served as a referral centre for specimens from the Kantha Bopha hospitals in Phnom Penh. The study period from 1 January 2004 to 15 July 2008 was divided into three phases: *Phase 1*: from 1 January 2004 to 30 June 2005, establishing laboratory infrastructure, procedures, and logistics. *Phase 2*: from 1 July 2005 to 31 March 2006, all children hospitalized with a clinical diagnosis of TB were systematically evaluated. *Phase 3*: from 1 April to 15 July 2008, sampling was limited to children with microbiologically confirmed TB in order to expand a *M. tuberculosis* isolate collection. Sampling in Phnom Penh was not as systematic as in Siem Reap.

Ethical considerations

At admission to the hospital, the child's guardian is requested to provide informed and signed (fingerprint) consent for the necessary diagnostic and treatment procedures. This record is kept as a permanent document in the patient's file.

METHODS

Basic laboratory examinations upon admission included haematology, biochemistry, and urine examinations in accordance with locally established hospital standards. Laboratory tests were performed after obtaining written consent from the child's guardian.

TB-specific laboratory examinations included collection of clinical specimens for microbiological examination as described previously [4]. Included were patients who had at least one positive result with any of the three methods (bright-field microscopy, rRNA amplification, culture) on at least one specimen. Drug susceptibility testing and genotyping were performed from strains isolated by culture.

Specimens were homogenized, decontaminated, neutralized, and centrifuged as described previously [4]. One aliquot was stored at +4–8 °C locally for up to 6 months after addition of the antimicrobial cocktail PANTA™ (BD, USA) to 0·5% Middlebrook 7H10 agar (Difco, USA) and sent in batches to the collaborating Institute of Infectious Diseases in Bern, Switzerland for culture, drug susceptibility testing, and genotyping.

Culture

One slant of Löwenstein–Jensen medium containing pyruvate and one BacT/ALERT Mycobacteria bottle with Mycobacteria antibiotic supplement (bioMérieux, Switzerland) were inoculated with 0·2 and 0·5 ml of the sediments, respectively. Slants of the solid medium were incubated at 35 °C in ambient air and BacT/ALERT Mycobacteria process bottles in the BacT/ALERT Microbial Detection System (bioMérieux) at 35 °C according to the manufacturer's recommendations, respectively. Incubation was up to 8 weeks. *M. tuberculosis* complex isolates were identified to the species level using the GenoType® MTBC assay (Hain Life Sciences GmbH, Germany).

Genotyping

The spoligotyping method was performed as described previously [9]. Biotin-labelled PCR products were detected by hybridization onto a spoligotyping membrane (Isogen Life Science, The Netherlands). Each test run included purified sterile water and *M. tuberculosis* control strain H37RV, and *M. bovis* BCG strain P3 controls. The results were analysed based on the standards provided by the SITVIT database (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp) [10]. This SpolDB4 international online database of spoligotype patterns has been established wherein a clustered pattern is designated a shared international type (SIT) [11]. During the later study phase the MIRU-VNTR*plus* database (http://www.miru-vntrplus.org) was used [12, 13]. 15-MIRU-VNTR typing followed the proposed standard approach [14]. DNA extraction from cultures was performed in Bern. Aliquots of a subset were typed independently in Bern and at the National Mycobacteria Reference Laboratory, National Institute for Public Health and the Environment, Bilthoven, The Netherlands.

Drug susceptibility testing

Drug susceptibilities were assessed using the radiometric method in a Bactec 460-TB instrument (PRISE, Becton Dickinson, Germany). The critical drug concentrations (in mg/l) were: pyrazinamide,

100; rifampicin, 2·0; isoniazid, 0·1; streptomycin, 2·0; and ethambutol, 2·5.

Electronic database and analysis

Data were abstracted from the paper documents as recorded by healthcare staff and captured in an EpiData relational database (EpiData Association, v. 3.1, Denmark). Data were analysed by EpiData Analysis, v. 2.2 (freely available at http://www.epidata.dk), OpenEpi (http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm) and Stata v. 12.1 (StataCorp, USA) as appropriate. For categorical variables we determined proportions with 95% confidence intervals (CIs). Where appropriate, we calculated odds ratios (ORs) with 95% CIs using the Robins–Breslow–Greenland method [15]. For continuous variables we used standard measures of central tendency such as means and percentiles.

We created categories based on frequencies of four spoligotypes: (1) the East African-Indian (EAI) genotype family, (2) the Beijing genotype family, (3) all others with a defined spoligotype, and (4) a group of undefined spoligotypes. We analysed factors that might be associated with each of these categories, examining other relevant strain characteristics (drug susceptibility), their frequency in time (study period), geographical distribution (patient's province of residence), and patient's characteristics (age, sex, disease site, HIV status).

A modified Poisson regression approach was applied to conduct univariable and multivariable analyses and to obtain relative prevalence estimates for the presence of any drug susceptibility [16]. In the multivariable regression we included the SIT and province group as these were the main characteristics of interest in the analysis of drug susceptibility. A P value <0·05 was considered statistically significant.

RESULTS

During the study period, 260 children aged <17 years were diagnosed with microbiologically confirmed TB (Table 1) [4]. Of these, 162 (62·3%) had TB confirmed by culture. In 161/162 patients, spoligotypes and drug susceptibility patterns were determined. The 161 children with *M. tuberculosis* isolates available are the subject of this report. These 161 children did not statistically differ by age and sex from the 99 not included.

Genotyping was independently performed by two laboratories (Bern and Bilthoven) and revealed identical results. One hundred of the 161 patients had a single isolate available for spoligotyping, 32 had two and 27 had three identical isolates. One child (Table 2, patient A) had three isolates, two were identical and one with a different spoligotype. Another child (Table 2, patient B) had five isolates; four were of the same spoligotype. The 161 children thus had 253 isolates and 163 strains. Because the two children with two different strains (as confirmed by MIRU-VNTR typing) belonged to the same 'undefined (orphan) spoligotype group', we could assign each of the 161 children to one single spoligotype group and use the child as the unit of analysis.

A first condensation provided 12 SIT groupings (Table 1). The most frequent spoligotype was EAI_SOM (n = 51, 31·7% of all spoligotypes), followed by 32 orphan SITs (19·9%). The next most frequent were EAI5, followed by the Beijing strain family, EAI2_NTB, and U. The EAI family accounted for the majority with 95 (59·0%, 95% CI 51·3–66·3) among all spoligotypes.

Drug susceptibility was tested for isoniazid, rifampicin, ethambutol, streptomycin, and pyrazinamide. There were no differences in the drug resistance pattern among the samples from each patient. Of the strains isolated from the 161 patients, 137 (85·1%) were susceptible to all five drugs (Table 1). *M. tuberculosis* from two children showed mono-resistance to pyrazinamide.

Grouping of patient provenance

The province of residence was known for 137 (85·1%) of the 161 children. Children who attended the services of the hospitals in Siem Reap and Phnom Penh originated from 16 of the 24 provinces in Cambodia covering three quarters of the country's population. Three groups of provenance were created: (1) the 'Kampong Thom group' with 35 children, (2) the 'Siem Reap group' with 87 children from Siem Reap, five of its neighbouring provinces (except Kampong Thom), and 11 children attending the hospital in Siem Reap but without information on province of residence, (3) 'the Phnom Penh group' with 39 children from Phnom Penh, eight of its neighbouring provinces, and 13 children attending the hospital in Phnom Penh but without information on province of residence (Fig. 1).

Univariable analysis of genotyping results

In the univariable analysis, two characteristics emerged as being associated with the spoligotype: the province group and the drug susceptibility result.

Table 1. *Characteristics of* Mycobacterium tuberculosis *strains among children by study phase, type of microbiological diagnosis, genotype, and drug susceptibility pattern. Cambodia, Kantha Bopha hospitals, 1 January 2004 to 15 July 2008*

Characteristic	Phase 1	Phase 2	Phase 3	Total	
				Number	col %
Microbiologically confirmed*	67	97	96	260	100·0
RNA pos., culture pos., Ziehl pos.†	***38***	***40***	***55***	***133***	51·2
RNA pos., culture pos., Ziehl neg.†	***2***	***4***	***19***	***25***	9·6
RNA neg., culture pos., Ziehl pos.†	***1***	***3***	***0***	***4***	1·5
RNA pos., culture neg., Ziehl pos.†	*8*	*11*	*8*	*27*	10·4
RNA pos., culture neg., Ziehl neg.†	*1*	*32*	*14*	*47*	18·1
RNA neg., culture neg., Ziehl pos.†	*17*	*7*	*0*	*24*	9·2
Culture-positive	41	47	74	162	62·3
Culture-positive with spoligotype and DST	41	46	74	161	100·0
Spoligotypes					
EAI5_SOM	11	14	26	51	31·7
Undefined	4	7	21	32	19·9
EAI5	11	6	8	25	15·5
Beijing	5	7	8	20	12·4
EAI2_NTB	3	7	7	17	10·6
U	2	4	4	10	6·2
T1	1	0	0	1	0·6
LAM9	1	0	0	1	0·6
H1	1	0	0	1	0·6
EAI4_VNM	1	0	0	1	0·6
EAI2_MANILA	1	0	0	1	0·6
MANU1	0	1	0	1	0·6
Drug susceptibility pattern‡					
- - - - -	31	40	66	137	85·1
H- - - -	1	1	2	4	2·5
HRE- -	0	0	1	1	0·6
HR- -Z	2	0	0	2	1·2
H- -S-	2	2	0	4	2·5
-R- - -	0	0	1	1	0·6
- - -S-	4	3	3	10	6·2
- - - -Z	1	0	1	2	1·2

H, Isoniazid; R, rifampicin; E, ethambutol; S, streptomycin; Z, pyrazinamide; DST, drug susceptibility test.
* RNA pos./neg., Positive/negative on rRNA amplification test; culture pos./neg., positive/negative on culture; Ziehl pos./neg., positive/negative on microscopy using the Ziehl–Neelsen technique.
† Percentages in last column refer to confirmed cases.
‡ - - - - -, Susceptible to all five drugs. The symbol for the drug interrupting the sequence of five hyphens indicates resistance to that drug.

Children from the Siem Reap group contained the EAI spoligotype significantly more frequently compared to children from the Phnom Penh group (Table 3). Conversely, children from the Phnom Penh group had the highest prevalence of the Beijing family spoligotype, and those from the Siem Reap group the lowest (trend not statistically significant, $P = 0{\cdot}06$).

Children with an EAI spoligotype were significantly more likely to have a drug-susceptible strain then children not having an EAI spoligotype. Focusing on the Beijing strain family, children with any drug resistance were more likely to have a Beijing spoligotype.

Univariable analysis of drug susceptibility testing results

Because pyrazinamide is not usually tested in drug surveys nor part of international proficiency test

Table 2. *Summary of the spoligotyping and 15-MIRU-VNTR typing in two children with* Mycobacterium tuberculosis *isolates obtained from sampling over four (patient A) and seven (patient B) consecutive days, respectively. In patient A the spoligotype and MIRU-VNTR typing results were identical in the samples obtained within the first 2 days following hospitalization, the spoligotype was different in the sample obtained at day 4 and there were differences at three genetic loci in MIRU-VNTR typing in the three samples available for testing. In patient B five samples were available; the spoligotype in sample 2 was different from four other isolates; there was one insignificant MIRU-VNTR type variation among all five samples available*

					MIRU-VNTR type													
Patient	Age (yr)	Sex	DST	Octal code	580	2996	802	960	1644	3192	424	577	2165	2401	3690	4156	2163b	1955	4652
A	12·2	M	Susceptible	777 777 7**7**7 **00**3 731	7	7	3	4	2	4	2	4	6	2	4	1	2	5	4
			Susceptible	777 777 7**7**7 **00**3 731	7	7	3	4	2	4	2	4	6	2	4	1	2	5	4
			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			Susceptible	777 777 7**3**7 **41**3 731	7	2	3	4	2	5	2	4	6	2	4	1	2	5	6
B	14·0	F	Susceptible	777 607 777 740 77**1**	2	2	5	2	1	3	2	4	2	2	5	2	2	3	5
			Susceptible	777 607 777 740 74**0**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			Susceptible	777 607 777 740 77**1**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			Susceptible	777 607 777 740 77**1**	2	7	5	2	1	3	2	4	2	2	5	2	2	3	5
			Susceptible	777 607 777 740 77**1**	2	2	5	2	1	3	2	4	2	2	5	2	2	3	5
			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

DST, Drug susceptibility test; n.d., not done; F, female, M, male.
In the Octal code, the gene(s) with differences in repeat testing are shown bold and underlined. In the MIRU-VNTR type, gene loci with difference on repeat testing are shown with a grey background.

Fig. 1. Map of Cambodia with 24 provinces/municipalities. Light grey shading: Siem Reap group; intermediate grey shading: Kampong Thom; dark grey shading: Phnom Penh group of provinces. The location of the Jayavarman VII Hospital in Siem Reap province is shown as a solid symbol (●). Cambodia, Kantha Bopha hospitals, 1 January 2004 to 15 July 2008.

panels and because ethambutol results are not very reliable [17], we focused our analyses on isoniazid, rifampicin, and streptomycin. Considering these drugs, 22 (13·7%, 95% CI 9·2–19·8) strains showed resistance against at least one drug, 14 (8·7%) to streptomycin, 11 (6·8%) to isoniazid, and three (1·9%, 95% CI 0·6–5·3) revealed multidrug resistance (resistance to at least isoniazid and rifampicin) (Table 4).

The prevalence of resistance by date of isolation, provenance, and person (age, sex, disease site, HIV serostatus) was also examined. Beijing spoligotype and the patient's provenance were significantly associated with any drug resistance, but none of the other factors.

Multivariable analysis

The results of the relative prevalence of any drug resistance by various patient characteristics in the univariable approach and the results of the adjusted multivariable analysis are summarized in Table 5. The variable 'HIV infection' was omitted from the analysis because the proportion of children with a missing result was large and precluded a meaningful analysis. The geographical gradient observed in the crude analysis was somewhat reduced when adjusted for spoligotype but remained strong.

To determine the type of drug resistance that was actually responsible for the gradient we used the above groups. Among these, we defined two subgroups with, respectively, (1) streptomycin resistance but no isoniazid resistance (10/14 cases with any streptomycin and 22 cases with any resistance) and (2) isoniazid resistance but not streptomycin resistance (7/11 with any isoniazid) cases (Table 1). The geographical gradient is largely attributable to streptomycin, but not to isoniazid resistance (Fig. 2). Because 10/14 streptomycin-resistant strains were mono-resistant and four had a combined resistance against isoniazid

Table 3. *Spoligotype groups of* Mycobacterium tuberculosis *strains, by time, geographical provenance of patients, and patient characteristics, Cambodia, Kantha Bopha hospitals, 1 January 2004 to 15 July 2008*

	EAI group		Undefined		Beijing		Other defined		
Characteristic	No.	Row %	No.	Row %	No.	Row %	No.	Row %	Total
Total	95	59·0	32	19·9	20	12·4	14	8·7	161
Any resistance									
Fully susceptible	89	64·0	27	19·4	12	8·6	11	7·9	139
Any resistance	6	27·3	5	22·7	8	36·4	3	13·6	22
Enrolment phase									
1 Jan. 2004 to 30 June 2005	27	65·9	4	9·8	5	12·2	5	12·2	41
1 July 2005 to 31 Mar. 2006	27	58·7	7	15·2	7	15·2	5	10·9	46
1 Apr. 2006 to 15 July 2008	41	55·4	21	28·4	8	10·8	4	5·4	74
Province group									
Siem Reap group	61	70·1	13	14·9	7	8·0	6	6·9	87
Kampong Thom	16	45·7	12	34·3	4	11·4	3	8·6	35
Phnom Penh group	18	46·2	7	17·9	9	23·1	5	12·8	39
Age quartile (years)									
0 to <6·6	20	50·0	10	25·0	6	15·0	4	10·0	40
6·6 to <10·9	23	59·0	9	23·1	4	10·3	3	7·7	39
10·9 to <13·1	23	57·5	7	17·5	5	12·5	5	12·5	40
⩾13·1	29	69·0	6	14·3	5	11·9	2	4·8	42
Patient's sex									
Female	44	57·1	12	15·6	13	16·9	8	10·4	77
Male	50	64·1	18	23·1	6	7·7	4	5·1	78
Missing	1	16·7	2	33·3	1	16·7	2	33·3	6
Disease site									
Intrathoracic only	41	61·2	13	19·4	8	11·9	5	7·5	67
Extrathoracic only	17	51·5	8	24·2	6	18·2	2	6·1	33
Intra- and extrathoracic	37	60·7	11	18·0	6	9·8	7	11·5	61
HIV test result									
Negative	42	61·8	16	23·5	7	10·3	3	4·4	68
Positive	3	100·0	0	0·0	0	0·0	0	0·0	3
Missing	50	55·6	16	17·8	13	14·4	11	12·2	90

and streptomycin, the influence of streptomycin resistance on any resistance is substantial. Streptomycin resistance was also significantly associated with strain family: 4/10 streptomycin mono-resistant strains were of the Beijing genotype, while only 16/151 other strains were (OR 5·6, 95% CI 1·4–22·1). Adjusted for province group, the OR was slightly lower (3·7). Due to the small sample size, the confidence interval (0·77–18·1) was too wide to remain meaningful, but nevertheless suggested that the geographical gradient still held. There was no association between Beijing strains and isoniazid (but no streptomycin) resistance (OR 1·2).

DISCUSSION

TB in children reflects recent transmission patterns in the community, in contrast to adult TB. The *M. tuberculosis* genotype distribution among children mirrors the cumulative history of transmission up to the present time. Drug susceptibility patterns inform about both the recent past of drug use (e.g. streptomycin and isoniazid resistance) and current treatment quality (such as multidrug resistance).

The most conspicuous finding of *M. tuberculosis* strain characteristics was a geographical gradient roughly from the southeast to the northwest. In this direction, both drug resistance, notably streptomycin resistance, and the contribution of the Beijing strain family to morbidity decreased. Conversely, the contribution of the EAI spoligotype family to the province group-specific strains increased. Strain family and region were independently associated with drug resistance in the multivariable analysis. In the Mekong river delta in Viet Nam, overall a lower transmissibility

Table 4. *Drug susceptibility test results for isoniazid, rifampicin, and streptomycin only, by time, geographical provenance of patients, and patient characteristics, Cambodia, Kantha Bopha hospitals, 1 January 2004 to 15 July 2008*

	Any resistance		Any SM resistance		Any INH resistance		Any RMP resistance		Any MDR		Fully susceptible		
Characteristic	No.	Row %	No.	Row %	No.	Row %	No.	Row %	No.	Row %	No.	Row %	Total
Total	22	13·7	14	8·7	11	6·8	4	2·5	3	1·9	139	86·3	161
Shared international type													
EAI group	6	6·3	4	4·2	3	3·2	1	1·1	1	1·1	89	93·7	95
Undefined	5	15·6	1	3·1	3	9·4	2	6·3	1	3·1	27	84·4	32
Beijing group	8	40·0	7	35·0	4	20·0	0	0·0	0	0·0	12	60·0	20
Other defined	3	21·4	2	14·3	1	7·1	1	7·1	1	7·1	11	78·6	14
Enrolment phase													
1 Jan. 2004 to 30 June 2005	9	22·0	6	14·6	5	12·2	2	4·9	2	4·9	32	78·0	41
1 July 2005 to 31 Mar. 2006	6	13·0	5	10·9	3	6·5	0	0·0	0	0·0	40	87·0	46
1 Apr. 2006 to 15 July 2008	7	9·5	3	4·1	3	4·1	2	2·7	1	1·4	67	90·5	74
Province group													
Siem Reap group	4	4·6	1	1·1	3	3·4	1	1·1	1	1·1	83	95·4	87
Kampong Thom	6	17·1	4	11·4	3	8·6	0	0·0	0	0·0	29	82·9	35
Phnom Penh group	12	30·8	9	23·1	5	12·8	3	7·7	2	5·1	27	69·2	39
Age group (years)													
0 to <6·6	8	20·0	5	12·5	4	10·0	1	2·5	1	2·5	32	80·0	40
6·6 to <10·9	2	5·1	1	2·6	0	0·0	1	2·6	0	0·0	37	94·9	39
10·9 to <13·1	6	15·0	4	10·0	3	7·5	2	5·0	2	5·0	34	85·0	40
⩾13·1	6	14·3	4	9·5	4	9·5	0	0·0	0	0·0	36	85·7	42
Sex													
Female	13	16·9	9	11·7	7	9·1	2	2·6	2	2·6	64	83·1	77
Male	7	9·0	4	5·1	4	5·1	1	1·3	1	1·3	71	91·0	78
Missing	2	33·3	1	16·7	0	0·0	1	16·7	0	0·0	4	66·7	6
Disease site													
Intrathoracic only	6	9·0	6	9·0	0	0·0	0	0·0	0	0·0	61	91·0	67
Extrathoracic only	7	21·2	4	12·1	5	15·2	1	3·0	0	0·0	26	78·8	33
Intra- and extrathoracic	9	14·8	4	6·6	6	9·8	3	4·9	3	4·9	52	85·2	61
HIV test result													
Negative	4	5·9	2	2·9	2	2·9	0	0·0	0	0·0	64	94·1	68
Positive	1	33·3	0	0·0	1	33·3	1	33·3	1	33·3	2	66·7	3
Missing	17	18·9	12	13·3	8	8·9	3	3·3	2	2·2	73	81·1	90

SM, Streptomycin; INH, isoniazid; RMP, rifampicin; MDR, multidrug resistance; EAI, East African-Indian.

Table 5. *Univariable and multivariable analysis of relative prevalence of any anti-tuberculosis drug resistance by strain and patient characteristics. Cambodia, Kantha Bopha hospitals, 1 January 2004 to 15 July 2008*

				Univariable association			Multivariable association		
	Any resistance			Relative prevalence			Relative prevalence		
Characteristic	No.	Row %	Total	Point	95% CI	*P* value	Point	95% CI	*P* value
Total	22	13·7	161						
Shared international type									
EAI group	6	6·3	95	1		0·001	1		0·049
Undefined	5	15·6	32	2·5	(0·81–7·6)		2·0	(0·64–6·0)	
Beijing	8	40·0	20	6·3	(2·5–16·3)		4·2	(1·5–11·7)	
Other defined	3	21·4	14	3·4	(0·95–12·1)		2·5	(0·75–8·3)	
Enrolment phase									
1 Jan. 2004 to 30 June 2005	9	22·0	41	1		0·173			
1 July 2005 to 31 Mar. 2006	6	13·0	46	0·59	(0·23–1·5)				
1 Apr. 2006 to 15 July 2008	7	9·5	74	0·43	(0·17–1·1)				
Province group									
Siem Reap group	4	4·6	87	1		<0·001	1		0·025
Kampong Thom	6	17·1	35	3·7	(1·12–12·5)		3·1	(0·92–10·3)	
Phnom Penh group	12	30·8	39	6·7	(2·3–19·5)		4·8	(1·5–14·9)	
Age group (years)									
0 to <6·6	8	20·0	40	1		0·279			
6·6 to <10·9	2	5·1	39	0·26	(0·06–1·1)				
10·9 to <13·1	6	15·0	40	0·75	(0·28–2·0)				
⩾13·1	6	14·3	42	0·71	(0·27–1·9)				
Sex									
Female	13	16·9	77	1		0·129			
Male	7	9·0	78	0·53	(0·22–1·3)				
Missing	2	33·3	6	2·0	(0·57–6·8)				
Disease site									
Respiratory only	6	9·0	67	1		0·161			
Non-respiratory only	7	21·2	33	2·4	(0·86–6·5)				
Respiratory and non-respiratory	9	14·8	61	1·6	(0·62–4·4)				

CI, Confidence interval; EAI, East African-Indian.

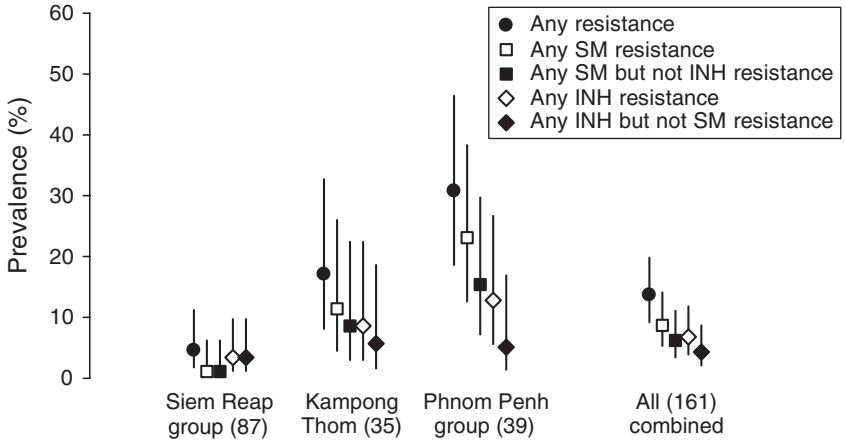

Fig. 2. Prevalence of drug resistance, against any drug, streptomycin, and isoniazid, by province group of patient's provenance. Symbols (circles, squares, diamonds) are point estimates; vertical lines denote 95% confidence intervals. Numbers in parentheses are number of child strains. Cambodia, Kantha Bopha hospitals, 1 January 2004 to 15 July 2008.

was shown for the Beijing genotype strains, showing relatively more fitness if streptomycin-resistant [18].

Among adults, a genotype analysis of strains from Phnom Penh has shown that among non-multidrug-resistant strains, Beijing genotypes accounted for 21% of the total [7] comparable to our 19% from roughly the same area.

Two children with different genotypes were identified among several isolates obtained from each child. In one child, the spoligotypes and corresponding 15-MIRU-VNTR types were substantially different, indicating a mixed infection. In the second child, the differing spoligotype can be explained by gene deletion [19, 20]. Since the introduction of genotyping methods, mixed infections among adults have become recognized more frequently than previously thought [21, 22] and have been estimated to occur at least in 3% of tuberculous adults in rural southern Viet Nam (neighbouring southern Cambodia) [23]. Mixed infections with *M. tuberculosis* in children have not previously been reported.

Epidemiological studies in children have the distinct advantage of reflecting characteristics of strains circulating in the community in the recent past. Strains isolated from adults point imprecisely to the acquisition time due to the ill-defined incubation period of TB [1].

On a comparative time axis, drug resistance points to a development in the recent past, while genotypes mirror a longer evolutionary past [24]. Nevertheless, there might be an intersection between drug resistance and genotype, the former may differentially affect the fitness of different genotypes, therefore the penetrating power of certain newly emerging strains may be superior [25–27].

Our analysis suggests that the observed geographical gradient was coincident rather than one confounding the other. On the shorter time axis, chemotherapy, as prerequisite for emergence of resistance, is likely to have been present in large urban settings prior to reaching rural settings at a time before there was a national TB programme. In Cambodia, the emergence of isoniazid resistance may have been facilitated by statements of the 1964 World Health Organization Expert Committee that was permissive to isoniazid monotherapy during the treatment continuation phase [28]. The problem of emerging drug resistance was subsequently compounded by recommendations to utilize isoniazid plus streptomycin (supplemented by pyrazinamide during the intensive phase), a widely used regimen in Cambodia well into the 1990s [29]. It is thus not surprising that a substantial amount of streptomycin resistance in adults remains as seen in the national surveys [5, 6]. The more rational 6-month, rifampicin-throughout regimen used nowadays in the national programme has not yet been able to curtail transmission of streptomycin-resistant strains to the youngest generation. We note the association between streptomycin but not isoniazid resistance with the Beijing strain type, as being reported from the Mekong river delta in Viet Nam [30], where the antecedents of current chemotherapy regimens are similar to Cambodia [31].

The global pattern of the spoligotype families plays out on a much grander time scale than drug resistance [24]. It is conceivable, that changes in the genotypic strain composition can be observed to occur relatively rapidly in small geographical areas [32]. In a wider regional context, the Beijing strain family is highly prevalent in the Mekong river delta in Viet Nam and increases with younger age, suggesting its relatively rapid spread [33]. A similar observation has been reported from Thailand (the frequency of Beijing strains decreased with increasing distance from the capital [34]). In China, marked geographical variations of genotypes have been reported, the Beijing family being the most prevalent [35]. There is evidence from our paediatric population that the Beijing strain family has reached the capital and its surroundings, although to a much lesser extent than in the neighbouring countries. It is still far from displacing the EAI spoligotype that dominates in the country and the northwest in particular.

A major weakness of our study is that it was not embedded in a similarly representative population-based sampling scheme as the two national drug resistance surveys. Sampling in Siem Reap was rather systematic, while in the Phnom Penh hospitals it was less so. Thus, our overall estimate underestimates the true prevalence of resistance in the whole of the country because the higher prevalence setting of the Phnom Penh area is relatively underrepresented in our sample.

Drug susceptibility patterns of *M. tuberculosis* isolated from infants and children reflect the characteristics of most recently transmitted strains currently circulating within the population [1]. The deterioration of the situation in South Africa has been documented in such a way in an exemplary fashion [3]. It is exceedingly difficult to obtain clinical isolates from children to assess the resistance situation, in fact, they are often specifically excluded. In a large national drug resistance survey in 2000–2001 in Cambodia, including 734 strains from patients with sputum

smear-positive TB, fewer than 10% of participants were aged <25 years, and children were virtually entirely absent [5]. In the national prevalence survey 2002, 245 strains were tested for drug susceptibility [6] to isoniazid, rifampicin, ethambutol and streptomycin. Children aged <10 years were exempted from radiographic and bacteriological examinations because of the technical difficulties with these procedures in children.

The prevalence of any resistance in the 2000–2001 national drug resistance survey and the 2002 national TB survey was 7·8% and 11·3%, respectively. This compares to 13·7% (or 14·3% if we include ethambutol but not pyrazinamide to make it comparable) in the current survey. Although the differences were small, we note the higher point estimate among our children. In contrast to the two national surveys, our sample size was not geographically balanced. While there is thus a sampling bias between geographical areas, there is much less of a bias in the ascertainment of genotypes and resistance prevalence within a geographical region. This latter demonstrates that resistance prevalence around the capital is worrisomely higher than in the more peripheral areas that were relatively overrepresented in our study. The crude prevalence of drug resistance underestimates the problem among children in the country. A higher prevalence in children than adults for a drug like isoniazid that has always been used for all patients points to an increase in the magnitude of the problem over time [1, 36]

Cambodia is one of the 22 high-burden countries which comprise 80% of the estimated global TB incidence. Over the past decade, TB in Cambodia has declined [37]. Childhood TB might also decrease, particularly if case finding and treatment among adult sources of transmission can further be strengthened.

ACKNOWLEDGEMENTS

The dedication and support of the medical, nursing and laboratory staff as well as the administrative and technical staff of the Kantha Bopha hospitals complex in Siem Reap, contributing to the excellent operation of this study, is greatly appreciated and warrants special recognition. We thank personnel at the collaborating Institute of Infectious Diseases in Switzerland: Jasmin Portmann introduced the diagnostic technologies, assisted in building up the operation of the TB laboratory, and trained the technical staff. Annemarie Hilty was responsible for laboratory cultures. Doris Schopfer and Simon Lüthi were in charge of reagent production, planning and logistics of shipments and uninterrupted supply of material and equipment. We also thank Salome Seiffert from the Institute in Bern and Kristin Kremer of the National Institute for Public Health (RIVM) in Bilthoven, The Netherlands, for excellent technical support. The project was funded by a grant of the University of Bern and the Kantha Bopha Foundation.

DECLARATION OF INTEREST

None.

REFERENCES

1. **Rieder HL.** Drug-resistant tuberculosis: issues in epidemiology and challenges for public health [Editorial]. *Tubercle and Lung Disease* 1994; **75**: 321–323.
2. **Schaaf HS, *et al.*** Primary drug-resistant tuberculosis in children. *International Journal of Tuberculosis and Lung Disease* 2000; **4**: 1149–1155.
3. **Seddon JA, *et al.*** The evolving epidemic of drug-resistant tuberculosis among children in Cape Town, South Africa. *International Journal of Tuberculosis and Lung Disease* 2012; **16**: 928–933.
4. **Schopfer K, *et al.*** Laboratory diagnosis of tuberculosis in a large pediatric hospital in Cambodia. *International Journal of Tuberculosis and Lung Disease* 2012; **16**: 503–509.
5. **Yamada N, *et al.*** The National Drug Resistance Survey in Cambodia, 2000–2001. *International Journal of Tuberculosis and Lung Disease* 2007; **11**: 1321–1327.
6. **National Center for Tuberculosis and Leprosy Control.** National TB prevalence survey, 2002 Cambodia. Phnom Penh, 2005, pp. 77.
7. **Zhang J, *et al.*** A first assessment of the genetic diversity of *Mycobacterium tuberculosis* complex in Cambodia. *BMC Infectious Diseases* 2011, **11**: 42.
8. **Richner B.** Annual report of the Foundation of Children's Hospitals Kantha Bopha, Dr med. Beat Richner. Annual Report 2011 (http://www.beatrichner.ch/pdf/Jahresberichte). Accessed 14 March 2014.
9. **Kamerbeek J, *et al.*** Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *Journal of Clinical Microbiology* 1997; **35**: 907–914.
10. **Brudey K, *et al.*** *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiology* 2006, **6**: 23.
11. **Lazzarini LCO, *et al.*** *Mycobacterium tuberculosis* spoligotypes that may derive from mixed strain infections are revealed by a novel computational approach. *Infection Genetics and Evolution* 2012; **12**: 798–806.

12. **Allix-Béguec C, *et al.*** Evaluation and strategy of use of MIRU-VNTR*plus*, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. *Journal of Clinical Microbiology* 2008; **46**: 2692–2699.
13. **Weniger T, *et al.*** MIRU-VNTR*plus*: a web tool for polyphasic genotyping of *Mycobacterium tuberculosis* complex bacteria. *Nucleic Acids Research* 2010; **38** (online issue): W326–331.
14. **Supply P, *et al.*** Proposal for standardization of optimized mycobacterial interspersed repetitive unit variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* 2006; **44**: 4498–4510.
15. **Altman DG, *et al.*** *Statistics with Confidence*, 2nd edn. Bristol: BMJ Group, 2000, pp. 240.
16. **Zou G.** A modified Poisson regression approach to prospective studies with binary data. *American Journal of Epidemiology* 2004; **159**: 702–706.
17. **Van Deun A, *et al.*** Drug susceptibility testing proficiency in the network of supranational tuberculosis reference laboratories. *International Journal of Tuberculosis and Lung Disease* 2011; **15**: 116–124.
18. **Buu TN, *et al.*** Increased transmission of Mycobacterium tuberculosis Beijing genotype strains associated with resistance to streptomycin: a population-based study. *PLoS ONE* 2012; **7**: e42323.
19. **Niemann S, *et al.*** Double infection with a resistant and a multidrug-resistant strain of *Mycobacterium tuberculosis*. *Emerging Infectious Diseases* 2010; **6**: 548–551.
20. **Cohen T, *et al.*** Mixed-strain *Mycobacterium tuberculosis* infections and the implications for tuberculosis treatment and control. *Clinical Microbiology Reviews* 2012; **25**: 708–719.
21. **Behr MA.** Tuberculosis due to multiple strains. A concern for the patient? A concern for tuberculosis control? [Editorial]. *American Journal of Respiratory and Critical Care Medicine* 2004; **169**: 554–555.
22. **Van Rie A, *et al.*** Reinfection and mixed infection cause changing *Mycobacterium tuberculosis* drug-resistance patterns. *American Journal of Respiratory and Critical Care Medicine* 2005; **172**: 636–642.
23. **Huyen MNT, *et al.*** Mixed tuberculosis infections in rural south Vietnam. *Journal of Clinical Microbiology* 2012; **50**: 1586–1592.
24. **Hershberg R, *et al.*** High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. *PLoS Biology* 2008; **6**: e311.
25. **Anh DD, *et al.*** *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. *Emerging Infectious Diseases* 2000; **6**: 302–305.
26. **Borrell S, Gagneux S.** Infectiousness, reproductive fitness and evolution of drug-resistant *Mycobacterium tuberculosis* [State of the Art Series, No. 2: Drug-resistant tuberculosis]. *International Journal of Tuberculosis and Lung Disease* 2009; **13**: 1456–1466.
27. **Cobelens F.** Relative reproductive fitness of the W-Beijing genotype [Editorial]. *International Journal of Tuberculosis and Lung Disease* 2012; **16**: 287.
28. **World Health Organization.** WHO Expert Committee on Tuberculosis, Eighth Report. *Technical Report Series* 1964; **290**: 1–24.
29. **Japan Anti-Tuberculosis Association, World Health Organization Regional Office for the Western Pacific.** Epidemiological review of tuberculosis in the Western Pacific Region 1995. In: *Research Institute of Tuberculosis Japan, World Health Organization*, 1st edn. Tokyo: The Research Institute of Tuberculosis, Japan, 1995, pp. 158.
30. **Buu TN, *et al.*** The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. *International Journal of Tuberculosis and Lung Disease* 2009; **13**: 900–906.
31. **Huong NT.** Tuberculosis control in Vietnam: does DOTS do it? (thesis). Amsterdam, The Netherlands: University of Amsterdam, 2007, pp. 158.
32. **Caminero JA, *et al.*** Epidemiological evidence of the spread of a *Mycobacterium tuberculosis* strain of the Beijing genotype on Gran Canaria island. *American Journal of Respiratory and Critical Care Medicine* 2001; **164**: 1165–1170.
33. **Buu TN, *et al.*** *Mycobacterium tuberculosis* genotype and case notification rates, rural Vietnam, 2003–2006. *Emerging Infectious Diseases* 2009; **15**: 1570–1577.
34. **Rienthong D, *et al.*** Restriction fragment length polymorphism study of nationwide samples of *Mycobacterium tuberculosis* in Thailand, 1997–1998. *International Journal of Tuberculosis and Lung Disease* 2005; **9**: 576–581.
35. **Dong H, *et al.*** Spoligotypes of *Mycobacterium tuberculosis* from different provinces of China. *Journal of Clinical Microbiology* 2010; **48**: 4102–4106.
36. **Forssbohm M, Loddenkemper R, Rieder HL.** Isoniazid resistance among tuberculosis patients by birth cohort in Germany. *International Journal of Tuberculosis and Lung Disease* 2003; **7**: 973–979.
37. **World Health Organization.** Global tuberculosis report 2013. World Health Organization Document, 2013; WHO/HTM/TB/2013·11, pp. 1–289.